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Inorganic Carbon Uptake by *Chlamydomonas reinhardtii*[1]

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ABSTRACT

The rates of CO_2-dependent O_2 evolution by *Chlamydomonas reinhardtii*, grown with either air levels of CO_2 or air with 5% CO_2, were measured at varying external pH. Over a pH range of 4.5 to 8.5, the external concentration of CO_2 required for half-maximal rates of photosynthesis was constant, averaging 25 micromolar for cells grown with 5% CO_2. This is consistent with the hypothesis that these cells take up CO_2 but not HCO_3^- from the medium and that their CO_2 requirement for photosynthesis reflects the $K_m(CO_2)$ of ribulose bisphosphate carboxylase. Over a pH range of 4.5 to 9.5, cells grown with air required an external CO_2 concentration of only 0.4 to 3 micromolar for half-maximal rates of photosynthesis, consistent with a mechanism to accumulate external inorganic carbon in these cells. Air-grown cells can utilize external inorganic carbon efficiently even at pH 4.5 where the HCO_3^- concentration is very low (40 nanomolar). However, at high external pH, where HCO_3^- predominates, these cells cannot accumulate inorganic carbon as efficiently and require higher concentrations of $NaHCO_3$ to maintain their photosynthetic activity. These results imply that, at the plasma membrane, CO_2 is the permeant inorganic carbon species in air-grown cells as well as in cells grown on 5% CO_2. If active HCO_3^- accumulation is a step in CO_2 concentration by air-grown *Chlamydomonas*, it probably takes place in internal compartments of the cell and not at the plasmalemma.

Unicellular green algae, such as *Chlamydomonas reinhardtii*, when grown with air levels of CO_2 can rapidly assimilate low levels of added bicarbonate much more efficiently than cells that had been grown on air supplemented with 5% CO_2 (5). This increased affinity for CO_2 in the air-grown cells indicates that they were adapted to conditions where CO_2 was limiting. A similar adaptation has been seen in *Scenedesmus obliquus* (8), *Chlorella vulgaris* (9, 13), and *Anabaena variabilis* (16). This increased affinity for external inorganic carbon ($HCO_3^- + CO_2$) seems to be associated with the ability by the cells to concentrate these substrates internally to levels much higher than the external concentration (2). This has led to the proposal that the increased affinity of air-grown cells for external carbon is due to the induction of a bicarbonate pumping mechanism.

Some investigators have also postulated that CO_2 and not HCO_3^- is the carbon species that diffuses across the cell membrane and that the carbonic anhydrase in the periplasmic space maintains the CO_2 supply by facilitating the rate of HCO_3^- to CO_2 interconversion (14, 29). Adaptation to low CO_2 conditions is associated with the induction of carbonic anhydrase (10). Cells grown with 5% CO_2 have very low levels of carbonic anhydrase activity, whereas cells grown with air have high levels (20). In *C. reinhardtii* a large portion of the carbonic anhydrase associated with air-grown cells is localized in the periplasmic space (18). CO_2 diffusion alone does not account for the accumulation of inorganic carbon by air-grown cells nor how these cells can utilize external CO_2 at concentrations well below the $K_m(CO_2)$ of ribulose-P_2 carboxylase. For that, the concept of a HCO_3^- pump has been invoked.

In this paper we report the effect of external pH on the uptake of external inorganic carbon by measuring CO_2-dependent O_2 evolution during photosynthesis and the uptake of ^{14}C-inorganic carbon into cells that had been grown on either 5% CO_2 or air. By altering the external pH over the range of 4.5 to 9.5, the ratio of CO_2 to HCO_3^- in the external medium was greatly changed. By comparing CO_2-dependent O_2 evolution fixation and inorganic carbon uptake at low pH, where CO_2 is the predominant species, and at high pH, where HCO_3^- predominates, it has been possible to consider which species of inorganic carbon was taken up by the cell.

MATERIALS AND METHODS

Chlamydomonas reinhardtii, strain 2137+, a gift from Dr. M. Spalding, was grown in a phosphate-rich, NH_4NO_3 medium (28). Similar results were obtained with strain 90 from the algal collection at the University of Texas-Austin. During growth, 1 L of the algal culture in 3-L Fernbach flasks were continuously mixed on an Eberbach shaker while being illuminated at 20 to 25°C with 100 $\mu E\ m^{-2}\ s^{-1}$ and aerated with air or air supplemented with 3 to 5% CO_2. Cells were harvested in the middle part of the log phase of growth, which was about 48 h after inoculation, by centrifugation at 1000*g* for 5 min at 4°C. The cells were washed once by resuspending the pellet in 20 ml of water and sedimenting them again at 10,000*g* for 10 min. This pellet was then resuspended in a buffer containing 25 mM Hepes-KOH (pH 7.2) to a final concentration of 20% (w/v) and stored on ice. All cells were used within 3 h from the time of harvest.

Photosynthetic CO_2-dependent O_2 evolution was measured with a Rank Brothers oxygen electrode (2). Harvested cells were diluted from the concentrated cell suspension to a final concentration of 1% (w/v) (25 μg Chl/ml) in the buffers indicated in the table and figure legends. The buffers were prepared daily and were brought to the indicated pH by the addition of KOH. Prior to the addition of cells, the buffer was bubbled with N_2 to lower both the dissolved CO_2 and O_2. Four ml of the diluted cell suspension in the O_2 electrode chamber at 25°C was then illuminated with 700 $\mu E\ m^{-2}\ s^{-1}$ of 400 to 700 nm light.

Two methods were used to measure the $K_{0.5}(HCO_3^- + CO_2)$[2]

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[2] Abbreviations: $K_{0.5}(HCO_3^- + CO_2)$, the concentration of inorganic carbon ($HCO_3^- + CO_2$) required to maintain O_2 evolution at one-half its maximum rate; $K_{0.5}(CO_2)$, the CO_2 concentration when oxygen evolution is half-maximal; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*'-3-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; ribulose-P_2 carboxylase, ribulose 1,5-bisphosphate carboxylase.

for photosynthesis by these cells. The first method was to follow a single progress curve (30) for O_2 evolution after addition of about 3 times the amount of HCO_3^- needed for half-maximal rates of photosynthesis. The $HCO_3^- + CO_2$ concentration when the O_2 evolution rate was 50% of maximum was then calculated. The HCO_3^- and CO_2 concentrations were calibrated by adding known amounts of $NaHCO_3$ and determining the extent of total O_2 evolution. These figures agreed with the O_2 calibration of the electrode indicating the O_2/HCO_3^- net exchange ratio was about 1. The second method was to add known amounts of $NaHCO_3$ and calculate the initial rate of oxygen evolution for each concentration. The two methods gave similar results. Before the addition of $NaHCO_3$ in either assay, the endogenous CO_2 was depleted by illuminating the algae until oxygen evolution ceased. For experiments at high pH (greater than 8.0) with cells grown on 5% CO_2, the time necessary to deplete the CO_2 was very long (>15 min). In these cases the $K_{0.5}(HCO_3^- + CO_2)$ was determined by the second method. The endogenous CO_2 levels were measured by illuminating a duplicate sample until the CO_2 was depleted and using this value to determine the total $NaHCO_3$ in the experiment.

For CO_2 compensation point determinations, a suspension of algae was diluted to 20 μg Chl/ml in the buffers indicated in Table II. The closed system was first flushed with 50 $\mu l \cdot l^{-1}$ CO_2, which allowed the high CO_2-grown cells to reach their compensation point within 15 min to minimize their adaptation to low CO_2 conditions. The atmosphere in the closed system was circulated by means of a diaphram pump, and the CO_2 content measured with a Beckman IR CO_2 analyzer (22).

The accumulation of inorganic carbon by the algal cells was estimated by the silicone oil filtration technique (2, 12). Assays were performed in the light (400 $\mu E\ m^{-2}\ s^{-1}$) at 25°C in 400 μl microfuge tubes in a Beckman Microfuge 11. The tubes contained (from bottom to top): 25 μl of 1 M glycine (pH 10.0) with 0.75% (w/v) SDS; 65 μl of silicone oil (1:1 [v/v] of Wäcker AR20 and Wäcker AR 200); 280 μl of the algal suspension that had been previously illuminated to deplete the cells of CO_2; and 30 μl of silicone oil (510 Dow Corning oil, from William F. Nye Inc, Bedford, MA). The top layer of silicone oil was added to reduce the loss of CO_2 by diffusion at the more acidic conditions. In addition, the microfuge tubes were sealed with a cap containing a small hole to allow additions by a syringe. Incubations were initiated by the injection of $NaH^{14}CO_3$ into the algal suspension to the final concentrations indicated. The reaction was terminated by centrifuging for 15 s. To allow the centrifugation to proceed in the light, the switch connected to the door latch was disconnected and a plexiglass sheet placed on top of the centrifuge.

Internal inorganic carbon was estimated from the difference between the alkaline and acid-stable ^{14}C in the pellet (2). The intracellular volume was estimated using [^{14}C]sorbitol and ^3H_2O (12). This volume was determined at each pH tested, but no significant changes in volume were observed. These numbers were then averaged and used to calculate the intracellular inorganic carbon concentration. Chl was determined spectrophotometrically (1).

RESULTS

$K_{0.5}$ (HCO_3 + CO_2). The concentration of total inorganic carbon ($HCO_3^- + CO_2$) required for half-maximal rates of photosynthesis at different pH values was determined with both high CO_2-grown or air-grown cells of *Chlamydomonas* (Table I). The concentration of HCO_3^- and CO_2 in the media of 25 mM buffers was changed by varying the pH between pH 5.95 and 8.45. The external pH and the different buffers had no effect on the maximum rates of oxygen evolution. The maximum photosynthetic rates were 140 μmol O_2 evolved $\cdot h^{-1} \cdot$ mg Chl^{-1} for high CO_2-grown cells and 115 μmol $h^{-1} \cdot$ mg Chl^{-1} for air-grown cells. While the maximum rates were not affected, the external pH caused a dramatic change in the measured $K_{0.5}(HCO_3^- + CO_2)$ for photosynthetic O_2 evolution by these cells (Table I; Figure 1). Cells grown on air were always more efficient (lower $K_{0.5}(HCO_3^- + CO_2)$) at using added inorganic carbon than cells grown on high CO_2. The calculated CO_2 concentrations when the photosynthesis rate was half-maximal at each external pH are presented in Table I. For cells grown on 5% CO_2 (in spite of the high $K_{0.5}(HCO_3^- + CO_2)$) under basic conditions, the calculated CO_2 concentration remained constant, averaging 23 μM, over the pH range examined. While this $K_{0.5}(CO_2)$ remained constant, the bicarbonate concentration required for half-maximal photosynthesis increased logarithmically with increasing pH (Table I). This lack of correlation between the external bicarbonate concentration and CO_2-dependent O_2 evolution has previously been observed (2) and interpreted to indicate that cells grown on high CO_2 cannot take up bicarbonate from the medium, *i.e.* they do not have an inorganic carbon pump.

In contrast to high CO_2-grown cells, *Chlamydomonas* cells grown with air had very low $K_{0.5}(HCO_3^- + CO_2)$ values for photosynthetic O_2 evolution of about 6 μM at pH 7.5 and values about 100 times smaller at pH 8.5 than those of high CO_2-grown cells. The calculated CO_2 concentration at $K_{0.5}$ was less than 1 μM at pH over 6. These low values, when compared with the high values for the algae grown with 5% CO_2, have been interpreted to indicate that air-grown cells have an inorganic carbon pump.

CO_2 Compensation Points. The CO_2 concentrations calculated in Table I for $K_{0.5}$ did not take into account the CO_2 in the algal suspension before adding $NaHCO_3$ for the measurement of O_2 evolution. The endogenous CO_2 remaining after the cessation of O_2 evolution and before adding a known amount of bicarbonate would be at the CO_2 compensation concentration. Since the CO_2 compensation point might change with external pH and growth conditions for the algae, and thus effect the $K_{0.5}$ calculations, the compensation point was determined for both air-grown and 5% CO_2-grown cells at pH 5.8 and 7.5 (Table II). A high compensation point of 35 or 56 $\mu l\ CO_2/l$ or 1 to 2 μM CO_2 was measured for the high CO_2-grown cells, and this range was close to that of a terrestrial C_3 plant. These high values probably reflect the lack of a CO_2 concentrating mechanism in high CO_2-grown cells. Thus, the average $K_{0.5}(CO_2)$ for photosynthesis by the high CO_2-grown cells should be increased from about 23 μM (Table I) to 25 μM. The CO_2 requirement for photosynthesis in these cells was close to the $K_m(CO_2)$ for ribulose-P_2 carboxylase, which has been reported to be 29 μM (15), although a higher value (55 μM) has also been reported (5).

With the air-grown cells, a very low CO_2 compensation point of less than 4 $\mu l \cdot l^{-1}$ or 0.14 μM CO_2 was observed (Table II). This value at pH 7.5 agrees with previous reports (6, 25). No change was seen in the compensation point at the lower pH of 5.8. In addition, the cells at pH 5.8 were depleting the medium of CO_2 at the same rate as the cells at pH 7.5. These data suggest that external HCO_3^- may not be required for concentrating CO_2 in these cells, since the HCO_3^- concentration at pH 5.8 in the presence of 4 $\mu l\ CO_2 \cdot l^{-1}$ should be about 40 nM. Since air-grown cells have high levels of carbonic anhydrase activity (10, 20), HCO_3^- and CO_2 are probably in equilibrium. These results disagree with the findings of Birmingham and Colman (6) who reported that the CO_2 compensation point increased at acidic pH for cells of *C. reinhardtii* and a number of other algae.

Effect of pH on $K_{0.5}$ Values for Photosynthesis. From the compensation point data the CO_2 concentrating mechanism in air-grown cells did not appear to be adversely affected by acidic external conditions. To further test this hypothesis, the $K_{0.5}(HCO_3^- + CO_2)$ for cells grown with air was measured over

Table I. *The External Carbon Concentration for Half-Maximum CO_2-Dependent O_2 Evolution at Different External pH*

The CO_2 and HCO_3^- concentrations at $K_{0.5}(HCO_3^- + CO_2)$ were calculated using a pK_a of 6.3.

Buffer	pH	5% CO_2-Grown Cells			Air-Grown Cells		
		$K_{0.5}(HCO_3^- + CO_2)$	$[CO_2]$	$[HCO_3^-]$	$K_{0.5}(HCO_3^- + CO_2)$	$[CO_2]$	$[HCO_3^-]$
		μM			μM		
25 mM Mes	5.95	35	24	11	4.0	2.8	1.2
25 mM Mes	6.45	62	25	37	1.8	0.75	1.1
25 mM Hepes	6.95	110	20	90	4.9	0.90	4.0
25 mM Hepes	7.45	350	23	327	5.8	0.38	5.4
25 mM EPPS	7.95	875	19	856	15	0.32	15
25 mM EPPS	8.45	3250	23	3227	27	0.21	27

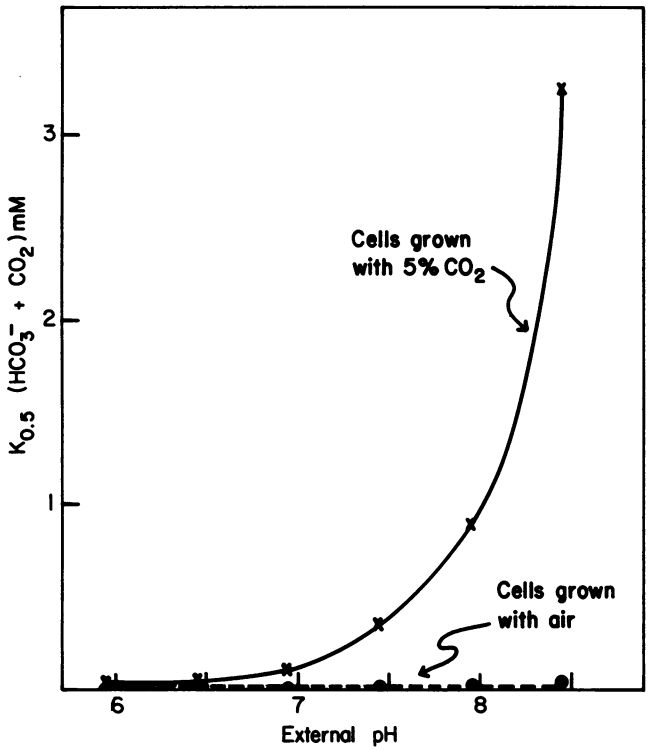

FIG. 1. pH dependence of $K_{0.5}(HCO_3^- + CO_2)$ for photosynthetic O_2 evolution by *Chlamydomonas*. Cells were grown either with 5% CO_2 (×) or air (●) and the $K_{0.5}(HCO_3^- + CO_2)$ determined by monitoring CO_2-dependent O_2 evolution.

Table II. *CO_2 Compensation Points for C. reinhardtii at pH 5.8 and 7.5*

Fifty ml of a cell suspension (20 μg Chl/ml) in either 25 mM Mes-KOH (pH 5.8) or 25 mM Hepes-KOH (pH 7.5) were allowed to deplete the CO_2 in a closed system in the light until the compensation point was reached. For 5% CO_2-grown cells the compensation point was reached within 15 min and was constant for the next 10 min. The air-grown cells were still slowly depleting the culture system of CO_2 after 30 min, but by then the CO_2 concentration was <0.14 μM.

Growth Conditions	pH	CO_2 Compensation Point	Calculated CO_2 Concn.
		$\mu l \cdot l^{-1}$	μM
5% CO_2	5.8	35	1.3
5% CO_2	7.5	56	2.1
Air	5.8	<4	<0.14
Air	7.5	<4	<0.14

a broader range of external pH (4.5–9.5). The buffers used and the kinetic values are given in Table III. V_{max} for photosynthetic O_2 evolution was constant between pH 4.5 and 9.5, but the concentration of HCO_3^- plus CO_2 required for half-maximal rates of O_2 evolution increased greatly at higher pH. Above pH 7.0, the calculated CO_2 concentration in the external medium needed to sustain half-maximal photosynthesis remained constant at about 0.4 μM, while the HCO_3^- concentration increased logarithmically. Between pH 7 and 6, the required CO_2, concentration, while remaining low, did increase somewhat to about 2 to 3 μM. This rise has also been noted by Badger *et al.* (2), who interpreted the lower $K_{0.5}(CO_2)$ at more basic conditions to the contribution of HCO_3^- to the carbon supply for the cell. If this lower $K_{0.5}(CO_2)$ at pH 8.0 were due to a HCO_3^- requirement for the CO_2 concentrating mechanism to operate, the $K_{0.5}(CO_2)$ should have increased under more acidic conditions where the concentration of HCO_3^- became very small. A large rise was not seen even at pH 4.5 where the measured $K_{0.5}(HCO_3^- + CO_2)$ translates to a CO_2 concentration of 3 μM and a HCO_3^- concentration of about 40 nM. These data agree with the CO_2 compensation point data at low pH that indicate that very low external concentrations of HCO_3^- do not adversely affect the mechanism for inorganic carbon accumulation in these cells.

The logarithm of the CO_2 and HCO_3^- concentrations needed for half-maximal rates of O_2 evolution over a wide pH range has been plotted in Figure 2 for *Chlamydomonas* cells grown with either air or 5% CO_2 in air. The fact that the CO_2 concentration required for half-maximal rates of photosynthesis in these cells remained nearly constant indicates that CO_2 may be the permeant species in air-grown cells. Both 5% CO_2-grown cells and air-grown cells apparently used external CO_2, but the CO_2 concentration required was 50-fold higher in 5% CO_2-grown cells (Fig. 2), presumably because these cells had no CO_2 concentrating mechanism.

An experimental difficulty was the accuracy of measuring the very low $K_{0.5}(HCO_3^- + CO_2)$ for air-grown cells. With air-grown cells at high pH or 5% CO_2-grown cells at any pH, the $K_{0.5}(HCO_3^- + CO_2)$ for O_2 evolution can be reproducibly measured, and these values were corroborated with studies measuring $^{14}CO_2$ fixation (data not shown). When the $K_{0.5}(HCO_3^- + CO_2)$ was lower than 5 μM, as was the case for air-grown cells below pH 7, there were some uncertainties in the accuracy of the O_2 evolution measurements. With a 4-ml suspension of algae, there was a lag of 5 to 10 s in the O_2 evolving response to added HCO_3^-, presumably due to the diffusion of the newly evolved O_2 to the oxygen electrode. This lag meant that at very low HCO_3^- concentrations, O_2 evolution hardly reached a constant rate before the external CO_2 concentration became limiting. Even with very dilute Chl concentrations, the added HCO_3^- was depleted within seconds. These uncertainties made the slight increase in the CO_2 concentration required for half-maximal photosynthesis in air-grown cells below pH 7.5 ambiguous (Fig. 2). It is possible that

Table III. *Inorganic Carbon Concentrations for Photosynthesis by Air-Grown Cells over a pH Range of 4.5 to 9.5*

The CO_2 and HCO_3^- concentrations were calculated using a pK_a of 6.3. At pH 8.5 and 9.5, the HCO_3^- concentration was also corrected for carbonate using a pK_a of 10.0.

Buffer	pH	V_{max}	$K_{0.5}(HCO_3^- + CO_2)$	$[CO_2]$	$[HCO_3^-]$
		$\mu mol\ O_2 \cdot h^{-1} \cdot mg\ Chl^{-1}$		μM	
25 mM citrate	4.5	122	3.0	3.0	0.041
25 mM citrate	5.5	132	3.1	2.7	0.42
25 mM Mes	6.0	128	2.9	1.9	1.0
25 mM Mes	6.5	125	4.0	1.5	2.5
25 mM Hepes	7.5	130	6.4	0.40	6
25 mM EPPS	8.5	135	60	0.38	58
25 mM CHES	9.5	138	760	0.48	760

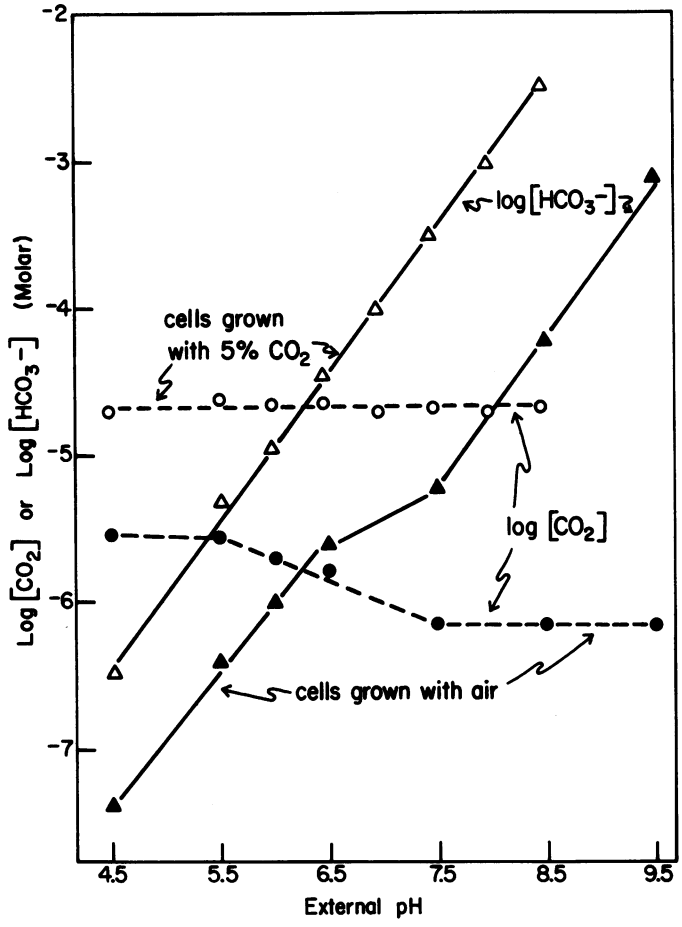

FIG. 2. The HCO_3^- concentration (△, ▲) or the CO_2 concentration (○, ●) required for half-maximal rates of O_2 evolution at varying pH. *Chlamydomonas* cells were grown with 5% CO_2 (△, ○) or with air (▲, ●). The buffers used at each pH are the same as shown in Table III. At pH 9.5, the concentration of HCO_3^- required for maximal rates of O_2 evolution in 5% CO_2-grown cells was very high (>100 mM) so the maximum rate could not be reliably measured. The HCO_3^- and CO_2 concentrations were calculated using a pK_a of 6.3.

the actual $K_{0.5}(HCO_3^- + CO_2)$ value below pH 6.0 is 0.5 μM which would be obtained by extrapolating from the more accurate data obtained at higher pH. The $K_{0.5}$ values reported in Table III at pH 6.0 and below should be viewed as the upper limits of the $K_{0.5}(HCO_3^- + CO_2)$. The method of single progress curves was used at low pH to avoid these difficulties and the results using this technique agreed well with the $K_{0.5}(HCO_3^- + CO_2)$ determined by measuring the O_2 evolution rates after addition of various concentrations of HCO_3^-.

Table IV. *The Accumulation of Inorganic Carbon by Chlamydomonas Cells Grown with Air or 5% CO_2*

Cells were illuminated in the indicated buffers until the endogenous CO_2 present was depleted to the level of the CO_2 compensation point. Inorganic carbon uptake was then measured as described in "Materials and Methods." The initial concentration of $NaH^{14}CO_3$ was 80 μM and the incubation time was 30 s. The calculated cellular volumes were 150 $\mu l \cdot mg\ Chl^{-1}$ for air-grown cells and 325 $\mu l \cdot mg\ Chl^{-1}$ for 5% CO_2-grown cells.

Buffer	pH	Intracellular $HCO_3^- + CO_2$	
		Cells grown with 5% CO_2	Cells grown with air
		mM	
25 mM citrate	4.5	0.40	1.02
25 mM Mes	6.0	0.27	1.05
25 mM Hepes	7.5	0.11	0.54
25 mM CHES	9.0	ND[a]	0.17

[a] Not determined. At pH 9.0, cells grown with 5% CO_2 could not deplete the endogenous CO_2 present in the buffers and did not evolve a measurable amount of O_2 when only 80 μM $NaHCO_3$ was added.

Uptake of Inorganic Carbon. This uptake was determined over a pH range of 4.5 to 9.0 (Table IV) by rapid separation of the cells from the medium in the light by centrifugation through a silicone oil layer. The data in Table IV are for inorganic carbon accumulation after 30 s at an external $NaHCO_3$ concentration of 80 μM. A time course of uptake was also measured and, as observed by Badger *et al.* (2), the rapid uptake cannot be resolved in air-grown cells, as it was the same between 10 and 60 s after the addition of $NaHCO_3$. Air-grown cells accumulated $HCO_3^- + CO_2$ even when the external pH was 4.5 and the HCO_3^- concentration was vanishingly small. The mechanism of this accumulation is unknown. Because the cells grown with 5% CO_2 did not have an inorganic carbon pump, their accumulation of inorganic carbon can be considered to occur by diffusion. At pH 7.2, the average internal pH of the cells has been calculated to be about 7.05 (2). If this internal pH were to remain constant even though the external pH was changed, a passive uptake to a concentration of between 0.4 and 0.5 mM would be expected when the external pH was 4.5 and the added inorganic carbon concentration was 80 μM. This is close to the inorganic carbon concentration found in the high CO_2-grown cells (Table IV). The extra accumulation by the air-grown cells may be due to a more basic interior of the cell or to the operation of a CO_2 concentration mechanism. In either case, air-grown cells accumulated inorganic carbon even at pH 4.5, which is consistent with their

low $K_{0.5}(HCO_3^- + CO_2)$ for O_2 evolution and a low CO_2 compensation point.

The concentration of inorganic carbon inside air-grown cells decreased as the external pH increased (Table IV). Air-grown cells accumulate inorganic carbon at pH 7.5 and 9.0 to internal concentrations higher than could be obtained by passive equilibration from the medium. However, the extent of accumulation was less at high pH and decreased as the external CO_2 concentration decreased due to the higher pH. The total inorganic carbon concentration was kept at 80 μM in these experiments. If the active accumulation was by a HCO_3^- pump at the plasmalemma, the opposite result would have been expected at higher pH, where more of the carbon existed as bicarbonate. These results are consistent with the hypothesis that CO_2 and not HCO_3^- is the carbon species taken up by these cells and that the internal concentration of inorganic carbon correlates with the photosynthesis rate (either O_2 evolution or $^{14}CO_2$ fixation). As the external pH increased, the amount of added $NaHCO_3$ required for half-maximal rates increased in both air-grown cells and 5% CO_2-grown cells (Table I).

DISCUSSION

Air-grown (low CO_2) algae utilize inorganic carbon more efficiently (5) and excrete less glycolate (17, 19) than cells grown with high CO_2. Two theories have been proposed to account for the ability of low CO_2-grown cells to utilize external inorganic carbon more efficiently: a HCO_3^- pumping mechanism (2) and a carbonic anhydrase mediated diffusion of CO_2 (14, 29). By varying the external pH, the CO_2 and HCO_3^- concentrations were altered over a wide range, and the rate of photosynthesis correlated with the CO_2 but not the HCO_3^- concentration. This supports the concept that CO_2 is the species taken up from the medium by air-grown *Chlamydomonas* cells (Fig. 3).

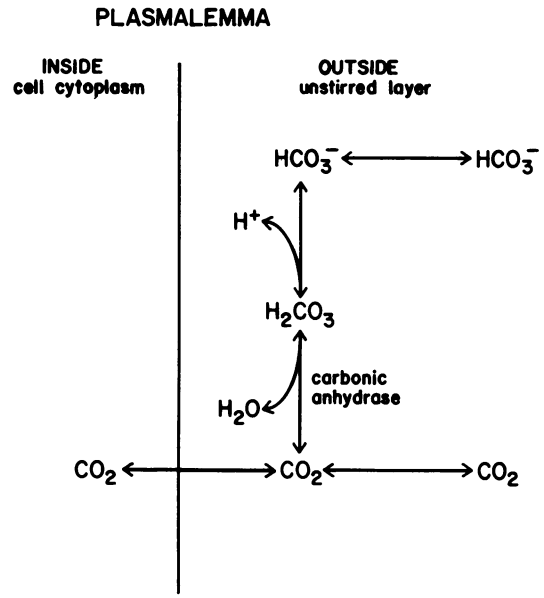

FIG. 3. Proposed scheme for CO_2 uptake by air-grown *Chlamydomonas*. CO_2 is the inorganic carbon species that crosses the plasma membrane. Under acidic conditions (HCO_3^- concentration low), CO_2 that enters the cell can only be replaced by CO_2 diffusing across the unstirred layer and this is the rate-limiting step. Under basic conditions (HCO_3^- concentration high), carbonic anhydrase effects the rapid replacement of CO_2 from HCO_3^- at the plasmalemma, thus bypassing this slow diffusional step. Because air-grown cells concentrate inorganic carbon, a mechanism for HCO_3^- accumulation and perhaps transport inside the cell or chloroplast is required (not shown in figure).

Previous work of Badger *et al.* (2) and Berry *et al.* (5) indicated that high CO_2-grown cells, without a CO_2 concentrating mechanism, use CO_2 from the medium but not HCO_3^-. Results in Table I and Figure 2 with *Chlamydomonas* cells grown with high CO_2 support this conclusion, in that the actual CO_2 concentration in the medium required for half-maximal photosynthesis changed very little over the pH range tested, while the HCO_3^- concentration followed the Henderson-Hasselbach equation by increasing logarithmically with pH.

The CO_2 and HCO_3^- requirements of the air-grown cells for photosynthesis were similar to those of high CO_2-grown cells in that these cells showed a constant CO_2 requirement for photosynthesis across the pH range, while the external HCO_3^- concentration was apparently immaterial. However, the CO_2 concentration required by air-grown cells was about 50 times less than that required by high CO_2-grown cells. This evidence indicates that CO_2 enters the air-grown cells rather than bicarbonate, and that an inorganic carbon accumulation mechanism is involved. Our interpretation of the results with air-grown cells is different than that of other workers who support a bicarbonate pump at the plasmalemma (2, 5). The evidence that CO_2, and not HCO_3^-, crosses the plasmalemma is 2-fold. First, under acidic conditions where the HCO_3^- concentration was exceedingly low (<40 nM), air-grown cells had (a) the same $K_{0.5}(HCO_3^- + CO_2)$ for photosynthesis, (b) a low CO_2 compensation point, and (c) retained the ability to accumulate inorganic carbon as at higher pH (>7). The $K_{0.5}(HCO_3^- + CO_2)$ for air-grown cells never approached the high value found for 5% CO_2-grown cells, even at pH 4.5, which might be expected if external HCO_3^- was required for the inorganic carbon concentrating mechanism. Second, as in 5% CO_2-grown cells, the rate of photosynthesis showed no dependence on the external HCO_3^- concentration across the pH range tested (Fig. 2). In fact, their ability to accumulate inorganic carbon decreased at high external pH (higher external HCO_3^-).

Studies with *Chlorella pyrenoidosa* indicated that CO_2 is the permeant inorganic carbon species in both high CO_2-grown and air-grown cells (4, 14, 23). Shelp and Canvin (23) showed that *Chlorella* had a low affinity for bicarbonate and concluded that a HCO_3^- pump was probably not in operation. If CO_2 is the inorganic carbon species that crosses the plasmalemma, yet an inorganic carbon concentrating mechanism occurs inside the air-grown cell, a high internal bicarbonate concentration or pump should exist, possibly at the level of the chloroplast envelope. This has been proposed by Beardall (3) from studies with the acid-tolerant *Chlorella saccharophilia*, which maintains the characteristics of an inorganic carbon concentrating mechanism even at pH 2.0 (3).

In *Chlamydomonas*, previous researchers have thought bicarbonate enters air-grown cells in addition to CO_2 because the $K_{0.5}(CO_2)$ decreases when the external pH is increased from 6.0 to 7.5 (2). Our data also show this decrease in the $K_{0.5}(CO_2)$ (Fig. 2). An alternate interpretation of this result is that at low pH (<6.0) the diffusion of CO_2 into the cell is rate limiting while at higher external pH another step involved in the accumulation of inorganic carbon is rate limiting. At high external pH the rate limitation due to CO_2 diffusion is overcome by the presence of HCO_3^- and carbonic anhydrase at the plasmalemma. Under these conditions CO_2 entering the cell can be rapidly replaced by dehydration of the excess HCO_3^- present. When the external pH is low, the only way the CO_2 that has entered the cell can be replaced is by diffusion of bulk CO_2 across the unstirred layer. This is potentially a slow step (7, 11, 21, 24). By having carbonic anhydrase in the periplasmic space, this slow step of CO_2 diffusion can be overcome when the external pH and bicarbonate concentration are high. Carbonic anhydrase has been shown to accelerate the diffusion of CO_2 across artificial bilayers at pH 7 to 8 by Gutknecht *et al.* (11). Therefore, it is likely that the

$K_{0.5}(CO_2)$ of 0.5 μM seen at pH 7.5 and above represents a rate-limiting step in the CO_2 accumulating mechanism while the higher $K_{0.5}(CO_2)$ of 3 μM seen below pH 6.0 is due to the slow step of diffusion of CO_2 across the unstirred layer.

The carbonic anhydrase located in the periplasmic space, however, would not account for the ability of air-grown cells to concentrate inorganic carbon. That periplasmic carbonic anhydrase is not responsible for the accumulation of inorganic carbon is supported by the work of Spalding *et al.* (25–27), who isolated mutants of *C. reinhardtii* that require high levels of CO_2 to grow phototrophically. One of these mutants cannot accumulate inorganic carbon yet has nearly wild-type levels of periplasmic carbonic anhydrase activity (26, and personal communication).

In summary, our pH experiments indicate that CO_2, but not HCO_3^-, permeates both 5% CO_2-grown and air-grown cells. In air-grown cells, a CO_2 concentrating mechanism is induced, so that only 0.4 to 2.5 μM external CO_2 is required for half maximum rates of photosynthesis, instead of the 20 to 30 μM CO_2 needed by cells grown on high CO_2 which lack his mechanism. Bicarbonate may still be accumulated within the cell, as in the chloroplast stroma, but if HCO_3^- is actively accumulated, this transporter may be located on the chloroplast envelope.

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